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REVIEWS

Improving growth, omega-3 contents, and disease resistance of Asian seabass: status of a 20-year family-based breeding program

**G. H. Yue · L. Wang · F. Sun · Z. T. Yang · J. Wong · Y. F. Wen ·
H. Y. Pang · M. Lee · S. T. Yeo · B. Liang · K. Chen · H. S. Lim · J. H. Jiang**

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Abstract Aquaculture has been one of the fastest-growing sectors in agriculture and plays an important role in supplying high quality proteins for humans. Genetic improvement for important traits is essential for increasing aquaculture production. The aquaculture of Asian seabass (*Lates calcarifer*) has become important in Southeast Asia and Australia and has expanded to other countries. In Singapore, a breeding program was initiated in 2004, aimed at improving growth rates, high omega-3 content, and disease resistance within Asian seabass populations. Many genomic resources have been developed to achieve these goals. The breeding program was established with a broodstock of 549 broodfish collected from the wilds of Indonesia, Thailand, Malaysia, and Singapore. Through four generations of family-based selection, utilizing a combination of conventional selective breeding, molecular parentage analysis, marker-assisted selection, and genomic selection techniques, three distinct elite lines of Asian seabass were successfully established. Each line consisted of approximately 200 broodfish. These lines were selected for growth, higher omega-3 content, and disease resistance, respectively. These traits have been improved without dramatically reducing genetic variation. This review provides a comprehensive overview of the methodologies adopted and status of the genetic improvement of the above-mentioned traits. Concurrently, certain gaps in the existing body of research have been identified. In the future, additional traits related to the ability to use feeds with reduced fishmeal, as well as adaptation to climate change and resistance against emerging diseases should be included in the breeding program.

Keywords Barramundi · Aquaculture · Breeding · MAS · GS · Trait

Introduction

Over the past thirty years, aquaculture has emerged as one of the fastest-growing food-producing sectors (Naylor et al. 2021). The main source of fish and

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G. H. Yue (✉) · L. Wang · F. Sun · Z. T. Yang · J. Wong · Y. F. Wen · H. Y. Pang · M. Lee · S. T. Yeo
Temasek Life Sciences Laboratory, National University of Singapore, 1 Research Link, Queenstown 117604, SG, Singapore
e-mail: genhua@tll.org.sg

G. H. Yue
Department of Biological Sciences, National University of Singapore, 14 Science Drive, Queenstown 117543, SG, Singapore

B. Liang · K. Chen · H. S. Lim · J. H. Jiang
Marine Aquaculture Center (MAC), Singapore Food Agency (SFA), 52 Jurong Gateway Road, #14-01, Jem 608550, SG, Singapore

shellfish for human diets is anticipated to be aquaculture (Garlock et al. 2022). In contrast with agricultural and livestock production, aquaculture production encompasses a wide variety of species that are diverse and are often only just beginning to be domesticated (Garlock et al. 2022; Naylor et al. 2021). To meet the nutritional demands of a rapidly expanding global population, particularly within underdeveloped regions, improvements in the scale and efficiency of aquaculture are required (Garlock et al. 2022; Kocour et al. 2010; Naylor et al. 2021). To improve aquaculture production, it is essential to develop improved breeds, achieved through carefully planned and managed breeding programs (Fu et al. 2022; Gjedrem 2005; Janssen et al. 2017; Rothbard et al. 2010). Breeding programs mainly rely on the mass selection and testing of candidate siblings to identify individuals with desirable traits. The ability to accurately predict the genetic potential of individuals at the early developmental stage could significantly enhance the genetic gain within a breeding program. Thus, the development of more accurate methods for early genetic prediction is crucial for the efficient selection of superior individuals (Gjedrem et al. 2012). With rapid advancements in DNA sequencing technologies and bioinformatics, genomic data have increasingly been used to maximize selective breeding for important traits within aquaculture species (Houston et al. 2020; Yue 2014). However, due to challenges related to reproduction, larval rearing, and post-captivity growth, selective breeding for marine finfish presents significant difficulties (Lucas et al. 2019; Nash 1977).

The Asian seabass (*Lates calcarifer*, Bloch), also known as barramundi, is a large, euryhaline member of the Latidae family. It is naturally distributed throughout the Indo-West Pacific region, encompassing Southeast Asian countries, China's Taiwan Province, northern Australia, the Arabian Gulf, and Papua New Guinea (FAO 2009). It has many characteristics that make it suitable for aquaculture. It is a hardy fish species, enabling it to withstand crowding. Additionally, it is able to tolerate a wide range of temperatures (16–36 °C) and salinity range (0–34 parts per thousand (PPT)) (FAO 2009). Female fish are highly fecund, with each of them producing over 1–40 million eggs for seed production in hatcheries, depending on body weight (Lim et al. 1986). This enables the establishment of robust seed production in hatcheries, thereby ensuring a stable supply of fingerlings for production. Various sizes of pelleted feed are available for this species. Juveniles are easy to wean on small-size pellets after 20–25 days post hatch (dph) (FAO 2009). The cultivation of Asian seabass has been a longstanding practice in Southeast Asia for over 70 years (Boonyaratpalin et al. 2002; Cheong and Yeng 1987). Presently, the Asian seabass has gained prominence as a key species for aquaculture in regions of Southeast Asia and Australia. Furthermore, the aquaculture of Asian seabass has also extended to China, India, South America, the Middle East, and various European countries (Ravisankar et al. 2010; Robinson et al. 2010; Stankus 2021). The Asian seabass has been cultured in Singapore since the 1980s (Cheong and Yeng 1987; Lim et al. 1986). This fish was suitable for aquaculture and had a good market acceptance (Cheong and Yeng 1987). Earlier research on Asian seabass focused on induced reproduction (Lim et al. 1986), larval culture (Sampath-Kumar et al. 1995; Sivaloganathan et al. 1998; Walford et al. 1993), and nutrition (Cuzon et al. 1989; Walford et al. 1991). These studies set a solid foundation to initiate a selective breeding program for Asian seabass. Since 2003, Temasek Life Sciences Laboratory (TLL) and Singapore Food Agency (SFA), with strong support from the government, have initiated a breeding program to improve growth, resistance against diseases, and meat quality (Shen et al. 2021). Other countries, including Vietnam (Khang et al. 2018), Thailand (Pattarapanyawong et al. 2021), Australia (Joerakate et al. 2018; Macbeth et al. 2011), Malaysia (Bakri and Esa 2021), and India (Behera et al. 2014) have also initiated breeding programs for Asian seabass. Many papers have been published on the various aspects of the Asian seabass, including studies related to its genetic diversity (Behera et al. 2014; Frost et al. 2006; Yue et al. 2009; Zhu et al. 2006), the utilization of genomic resources (Yue et al. 2023), and investigations into its biology (Cuzon et al. 1989; Moore 1979; Ye et al. 2017). These articles have been published in various journals, conference proceedings, and reports. Nonetheless, there is an evident lack of detailed information regarding the current status of Asian seabass breeding programs, despite some brief overviews detailing breeding programs for other aquaculture species in specific countries (Bakri and Esa 2021; Behera et al. 2014; Joerakate et al. 2018; Khang et al. 2018; Macbeth et al. 2011; Pattarapanyawong et al. 2021).

The aim of this review was to provide a comprehensive summary of the methodologies, current progress, results, and challenges within the Asian seabass breeding program in Singapore. We focused on the various aspects of the breeding program, including the establishment of the founder population, specific traits targeted for improvement, the selection methodologies, and performance of these traits. Furthermore, we highlighted several challenges and research gaps identified within the program. We proposed potential strategies to tackle the challenges and propel this program forward. We hope that the experiences and lessons learned from this breeding program will give readers some novel insights, enabling them to embark on their own breeding programs for other aquaculture species.

Status of Asian seabass aquaculture in the world

The Asian seabass has been an important aquaculture species in Southeast Asia since the 1950s (Boonyaratpalin et al. 2002; Cheong and Yeng 1987) and, presently, in Australia (FAO 2022; Loughnan et al. 2019). This species has been introduced to many regions and countries for aquaculture purposes (FAO 2022; Stankus 2021). At present, the annual global production of Asian seabass exceeds 105,000 tons (FAO 2022). It is estimated that the Asian seabass market will expand at a consistent annual growth rate of 5.5% until 2031 (Roy 2021). Given the importance of a reliable supply of high-quality seeds in aquaculture production, several countries have initiated selective breeding programs aiming at the genetic improvement of important traits for the Asian seabass (Bakri and Esa 2021; Behera et al. 2014; Joerakate et al. 2018; Khang et al. 2018; Macbeth et al. 2011; Pattarapanyawong et al. 2021).

The Asian seabass breeding program in Singapore

The Asian seabass breeding program in Singapore started in 2004 and is probably one of the longest-running programs focused on this species. Since 1998, numerous genomic resources and tools have been developed to facilitate the breeding program (Yue et al. 2023). A founder population of 549 broodfish with high genetic variation was established in January 2004 (Zhu et al. 2006). The targeted traits of the breeding program were growth, high omega-3 fatty acid content in meats, and resistance against major diseases in hatcheries (Liu et al. 2016c; Wang et al. 2017b; Xia et al. 2014; Xu et al. 2006). Several methodologies have been utilized in this program, including conventional selective breeding, which involves within family selection with the assistance of microsatellite markers for molecular parentage determination, marker-assisted selection (MAS), and genomic selection (GS) using DNA markers associated with specific traits. In the subsequent subsections, we provide comprehensive insights into several important aspects of this breeding program.

A founder population, genetic diversity, and breeding goals

Since the 1980s, the Asian seabass has been cultivated within a government hatchery located in Singapore for the purpose of commercial production (Cheong and Yeng 1987). After successfully closing the entire life cycle of Asian seabass (see Fig. 1E) in controlled aquaculture conditions (Lim et al. 1986), researchers proceeded to investigate different farming methods (Cheong and Yeng 1987). This included aspects such as the consistent production of young fish (seeds) through several spawning methods (Lim et al. 1986), and development of suitable feeding strategies (Cuzon et al. 1989). These studies found that cultivating Asian seabass is simpler compared to other marine species such as snappers and groupers. Moreover, the local community has shown a preference for consuming Asian seabass. Therefore, Singapore’s choice to prioritize Asian seabass within its aquaculture industry is a result of the species’ adaptability, growth characteristics, market demand, research efforts, and alignment with broader national objectives concerning food security and sustainable development. As a result, the Asian seabass was selected as the target fish species for genetic improvement within aquaculture in 2004. The initiation of a genetic improvement program, through selective and molecular breeding approaches, involved the procurement of a total of 549 adult Asian seabass from their respective wild populations in Singapore (n = 104), Thailand (n = 132), Indonesia (n = 148), and Malaysia (n = 165) (Zhu et al. 2006). They were brought to Singapore and cultured in the Marine Aquaculture Center (MAC), which is a governmental aquaculture

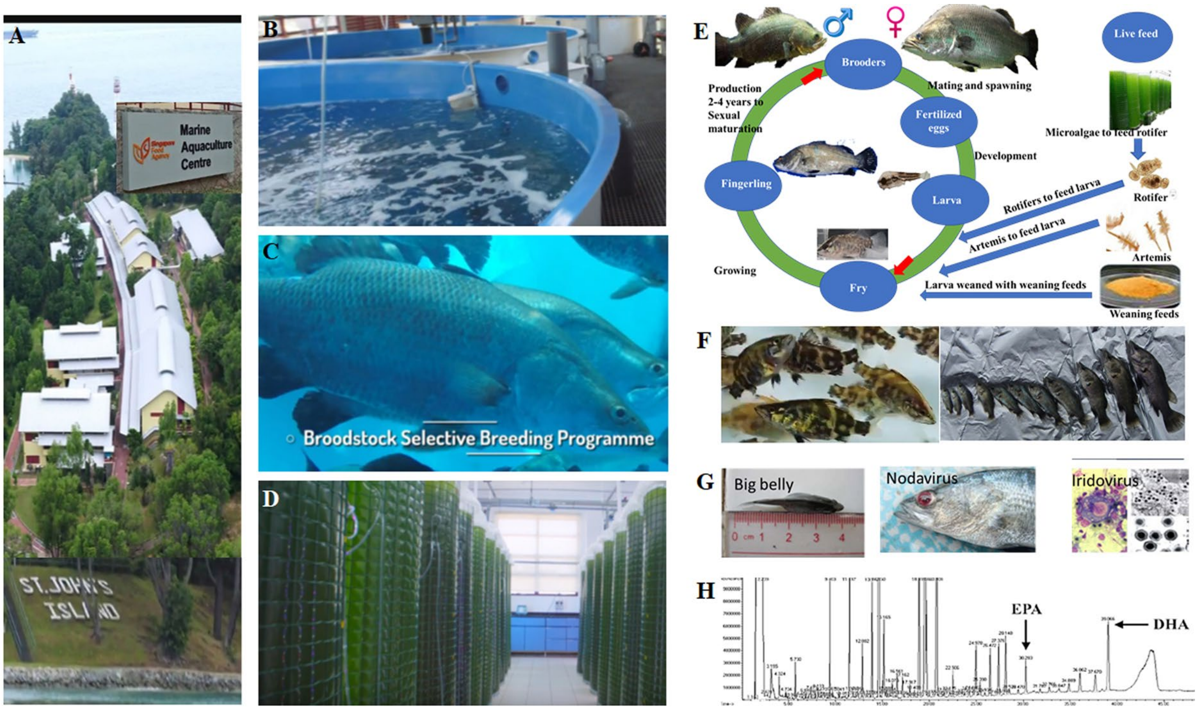

Fig. 1 Overview of the facilities for the Asian seabass breeding program conducted in the Marine Aquaculture Center, Singapore, and some information of the Asian seabass. **A**: Marine Aquaculture Center (MAC) was established in St. John's Island in 2003 to deepen Singapore's expertise in the areas of aquaculture genetics, nutrition, and health. **B**: 60-ton spawning tanks in MAC. **C**: Asian seabass broodfish in a spawning tank in MAC. **D**. Facilities to produce green algae and other live feeds for Asian seabass larvae. **E**: The closed life cycle of Asan seabass. **F**: Asian seabass larvae and fingerlings. **G**: Three major diseases caused by bacteria and viruses in hatcheries of Asian seabass. **H**: A fatty acid profile of an Asian seabass

research facility located on St. John's Island, Singapore (Fig. 1A). Fin clips were obtained from each of the 549 adult Asian seabass specimens, along with individuals from other cultured populations in Australia, Singapore, and Chinese Taiwan (Yue et al. 2009). Genotyping was performed on the 549 Asian seabass individuals, as well as those collected from Australian and Southeast Asian aquaculture farms, using polymorphic microsatellite markers (i.e., *par, Lca008, Lca020, Lca021, Lca058, Lca064, Lca069, Lca070, Lca074,* and *Lca098*) (Zhu et al. 2006). Wild stocks from Southeast Asia were found to be more genetically diverse as compared to those from Australia and Taiwan (Zhu et al. 2006). Therefore, the 549 individuals from the four wild populations in Southeast Asia were used as a founder population for the selective breeding program. Pairwise genetic relationships among the 549 adult broodfish were estimated based on the genotypes at the 10 microsatellite loci (Yue et al. 2009; Zhu et al. 2006). A phylogenetic tree showing the genetic relationships among the 549 adult broodfish was constructed based on genetic similarities estimated using the microsatellite genotypes. Figure 2 shows an overview of the breeding program of Asian seabass since 2004. Over the 20 year duration of the programme, the breeding goals have been expanded to improve an increasing number of traits, including growth, resistance to diseases, and omega-3 content. In F_1, the trait selected was growth performance. From F_2 onwards, traits such as disease resistance and omega-3 content were also selected.

Genomic resources for molecular breeding of Asian seabass

Since 1998, numerous genomic resources have been developed by our institute to accelerate the genetic improvement of Asian seabass (Yue et al. 2023). These genomic resources include microsatellite markers (Yue et al. 2001), single nucleotide

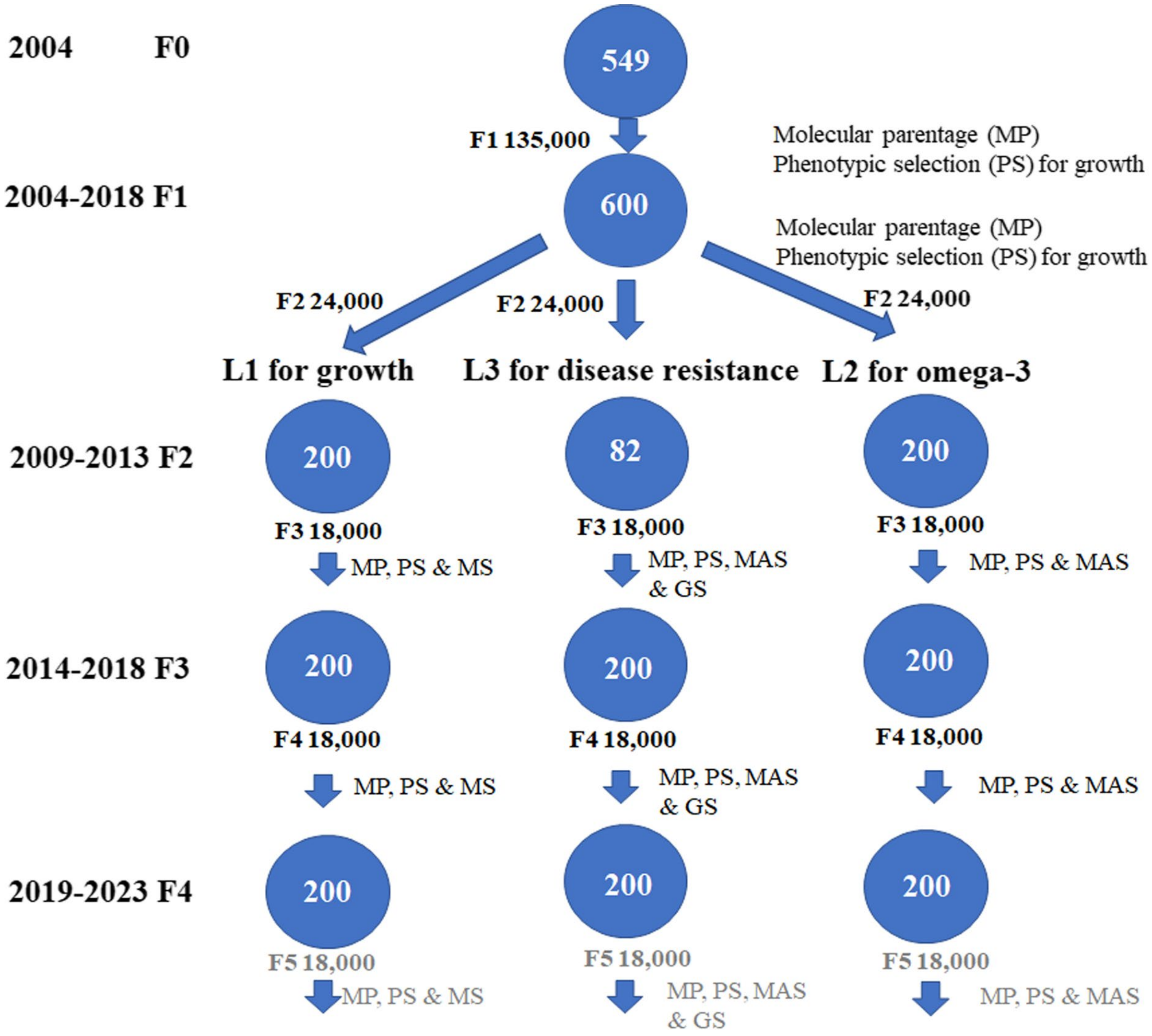

Fig. 2 Overview of selecting approaches for the 20-year Asian seabass breeding program in Singapore. The numbers in each cycle are the number of selected elite broodfish. F_0, F_1, F_2, F_3, and F_4: Generations from F_0 to F_4, and the numbers behind F_0, F_1, F_2, F_3, and F_4 are the numbers of fish raised for selection in each generation. L1, L2 and L3: Lines 1, 2 and 3. MS: Marker-assisted selection. GS: Genomic selection. The selection in F_5 will start in later 2023

polymorphisms (SNPs) located within genes (Xu et al. 2006) and spanning the whole genome (Wang et al. 2019), bacterial artificial chromosomes (BAC) and complementary DNA (cDNA) libraries (Wang et al. 2008b; Xia et al. 2010), along with genetic and physical maps (Wang et al. 2007, 2011, 2017c; Xia et al. 2010). These resources further encompass a system for genetic traceability (Yue et al. 2012), a molecular parentage system (Liu et al. 2012), transcriptomes (Liu et al. 2016b; Wang et al. 2021; Xia et al. 2010, 2011, 2013a), and draft genome sequences (Vij et al. 2016; Wang et al. 2016). These genomic resources have been used to accelerate genetic improvement for important traits, including growth (Xia et al. 2013b), omega-3 content (Sun et al. 2018; Wang et al. 2019; Xia et al. 2014) and disease resistance (Liu et al. 2016b; Liu et al. 2016c; Sun et al. 2020; Tay et al. 2023; Wang et al. 2017b; Yang et al. 2020; Yu et al. 2021). They have been used in many aspects of Asian seabass aquaculture, including estimations of genetic variation and population structure (Yue et al. 2009; Zhu et al. 2006), the reconstruction of pedigrees through parentage analysis (Liu et al. 2012), mapping of quantitative trait loci (QTL) (Wang et al. 2007, 2011, 2019) and genome-wide association studies (GWAS) (Wang et al. 2017a) for important traits, as well as applications in MAS, and GS. Detailed information on these genomic resources and their use in Asian seabass farming is available in a recent review (Yue et al. 2023). Here, we briefly describe the applications of genomic resources in molecular parentage determination, QTL mapping, and GWAS for important traits for the Asian seabass breeding program in Singapore.

Molecular parentage analysis is able to reconstruct pedigrees within mass-crosses of aquaculture species (Frost et al. 2006; Liu et al. 2012; O'Reilly et al. 2014; Yue et al. 2014). Pedigrees are essential for the estimation of genetic parameters (e.g., heritability) (Ye et al. 2017) and breeding values (Domingos et al. 2014). Due to the relatively small sizes of juvenile fish when compared to livestock, the use of conventional tagging methods is often challenging. Therefore, in aquaculture breeding programs, the young fish from each family must be raised separately until they are large enough for physical tagging. This method introduces additional environmental elements to the calculation of genetic parameters (e.g., heritability), in addition to being laborious and costly. In this Asian seabass breeding program, genotyping was conducted with 10 microsatellite markers on both offspring and parent candidates in each of the 105 mass crosses spanning from F_0 to F_4. This approach effectively assigned each offspring to their respective family groups, even in cases where various progeny groups had been reared collectively post-hatching (Liu et al. 2012). This reconstructed pedigree information enabled within-family selection in all the 105 mass crosses (Wong et al. 2023b).

QTL mapping is an essential step toward MAS (Yue 2014). A QTL is a chromosomal segment, which correlates with the variation observed in a quantitative trait within the phenotype of a population of organisms (Geldermann et al. 1985). QTL mapping involves the identification of molecular markers that exhibit correlation with a trait of interest. This is the first step towards the identification of causal genes responsible for the expression of the specific trait and the application of MAS (Yue and Wang 2017; Yue 2014). In this Asian seabass breeding program, QTL mapping has been used to identify DNA markers associated with growth (Wang et al. 2008a, 2007), omega-3 fatty acid content (Wang et al. 2019; Xia et al. 2014), and resistance to diseases caused by nodavirus (Liu et al. 2016a, 2016c) and iridovirus (Liu et al. 2016a, 2016c; Wang et al. 2017b). Some DNA markers associated with growth, omega-3 content, and disease resistance have been confirmed in different families or via association mapping (Sun et al. 2020; Sun et al. 2018; Tay et al. 2023; Xia et al. 2013b; Xu et al. 2006; Yang et al. 2020; Yu et al. 2021). These markers, which are significantly associated with traits, have gradually been used in MAS within the breeding program. This involves genotyping selection candidates and estimating their molecular breeding values (MBVs) based on genotype information. However, it is worth noting that most QTL with minor effects are family-specific and can only be used in families where the QTL were identified (Yue 2014). Therefore, only DNA markers located in QTL with major effects have been used in MAS within the selective breeding program. To capture DNA markers with minor effects on a phenotype of interest, more robust approaches such as GWAS, were used to identify associations between markers and corresponding traits.

A GWAS is a study on genotyping of numerous DNA markers (> 10,000), including SNPs and indels (deletion or insertion), in a large population (~ 1000 individuals) to identify the DNA markers that are associated with the trait of interest on the whole of the targeted species. DNA markers associated with traits of interest can be used in GS (Wang et al. 2005). In the Asian seabass breeding program, a GWAS was conducted targeting resistance against the nervous necrosis virus (NNV, i.e., nodavirus). The viral nervous necrosis disease (VNN) is a main risk to Asian seabass aquaculture, and is caused by NNV (Yang et al. 2022). In molecular breeding programs, the identification of DNA markers associated with NNV resistance and understanding the underlying mechanisms governing resistance to VNN are crucial to improving NNV resistance. By utilizing genotyping-by-sequencing (GBS), a GWAS was carried out to identify DNA markers associated with NNV resistance (Wang et al. 2017a). GBS was used to genotype a total of 986 fish from 43 families created by 15 founders and yielded 44,498 bi-allelic genetic variations. GWAS identified six loci with suggestive associations on LGs 1, 8, 14, 15, 21, and

24, and three genome-wide significant QTL on LGs 16, 19, and 20. These loci were found to be related to resistance to the NNV infection. A genome prediction accuracy of 0.7 was achieved by combining 800 training samples with 500 of the most significant markers. Some genes, including lysine-specific demethylase 2A, beta-defensin 1, and cystatin-B, which are key players in immune responses against viral infections, were among the candidate genes found to be strongly linked with resistance to NNV. Each one of the identified candidate genes showed differential expression in various organs in response to NNV infection. These genes were utilized in the understanding of the resistance mechanism against VNN. Subsequently, the 500 most significant markers were used for the selection of NNV-resistant individuals within the F_3 and F_4 population. Future research should utilize whole genome resequencing in conjunction with populations with large effective sizes, as this approach has the potential to search for additional beneficial genetic variations (Wang et al. 2017a).

Selection for growth

Within the aquaculture industry, growth traits are most significant as they directly determine the commercial value of fish (Gjedrem et al. 2012; Yue 2014). Consequently, within the Asian seabass breeding program, growth was the first trait selected for improvement. The estimated heritability for growth-related traits ranged from 0.24 to 0.34 (Wang et al. 2008c; Ye et al. 2017), suggesting that phenotypic selection for growth has the potential to yield effective results. Therefore, the initial stage of the Asia seabass breeding program involved the phenotypic selection aimed at improving growth.

Phenotypic selection within families in mass-crosses with the help of molecular parentage in F_1

Between 2004 and 2008, a total of 45 independent mass-crosses were conducted. Each mass-cross utilized 10–15 males and 10–15 females selected from the 549 broodfish (Fig. 3). In each breeding event, the chosen males and females were genetically distant

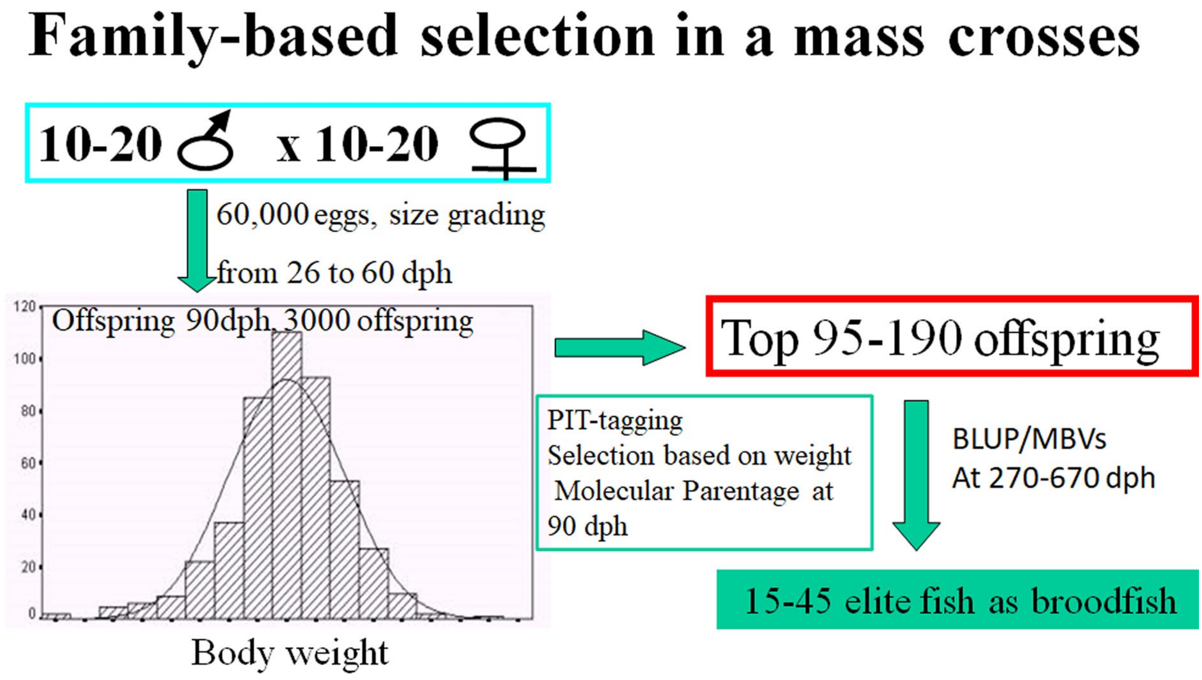

Fig. 3 Overview of family-based selection in each mass-cross in the breeding program of the Asian seabass *PIT* passive integrated transponders, *BLUP* best linear unbiased prediction, *MBVs* molecular breeding values estimated based on genotypes at loci associated with the traits of interest

from each other (Ye et al. 2017). After spawning, 450–1000 larvae from each mass-cross were sampled and genotyped with 10 polymorphic microsatellites. Using the genotype data from the broodfish and the collected larvae, the genetic parentage of the offspring was determined through the application of molecular parentage analysis techniques (Liu et al. 2012; Wang et al. 2008c; Yue et al. 2014; Zhu et al. 2010). In each mass-cross, 60,000 fertilized eggs were randomly collected from a pool of over two million fertilized eggs. These eggs were cultured according to a standard operating procedure (SOP). This egg selection process was used only when the number of families exceeded six. This was done to minimize any potential reduction of genetic diversity within the breeding population (Wang et al. 2008c; Ye et al. 2017). Grading was conducted weekly from 26 to 60 dph due to the cannibalistic nature of the Asian seabass. From each mass-cross, the largest 192 fish were selected from a pool of 3000 selection candidates at 90 dph. Each individual was tagged with an electronic PIT-tag and genotyped with 10 microsatellite markers. At 180 dph, fish were selected based on body weight, familial origin, and genotype profiles at the marker loci. Up to six individuals were selected from each family. At 2 years of age, a total of 600 superior F_1 were selected using breeding values estimated with the best linear unbiased prediction (BLUP) (Ye et al. 2017). These 600 F_1 fish were used as elite F_1 broodfish to produce F_2 for further selection for growth and other desirable traits.

Phenotypic and marker-assisted selection within families in mass-crosses in F_2, F_3 and F_4.

Between 2004 and 2008, while conducting the selection process for growth in the F_1 generation, a range of genomics resources, encompassing DNA markers and linkage maps, were developed (Wang et al. 2008a; Wang et al. 2007; Wang et al. 2008b; Xu et al. 2006; Yue et al. 2001; Yue et al. 2023). By using QTL mapping to assess growth traits across several families with different genetic backgrounds, the QTL associated with growth on specific LGs was detected in the tested families (Wang et al. 2008a, 2007; Xu et al. 2006). These DNA markers flanking the identified QTL regions, together with additional SNPs in relevant genes were used in MAS. Briefly, from 2009 to 2013, these 600 selected F_1 elite fish were used to produce the F_2 population, which were further selected for growth (Ye et al. 2017) and later for other traits (i.e., disease resistance). These 600 F_1 broodfish were divided into two lines, each made up of approximately 300 individuals, based on the genotypes at the 10 microsatellite loci (Ye et al. 2017). To prevent closely related family members from mating, the selection process for both males and females within each respective grouping was predicated on their genotypic dissimilarity across the 10 microsatellite loci. To produce each batch (i.e., offspring of a mass-cross) of F_2 fish, 15–20 male and female broodfish (i.e., up to 40 individuals) from the same brooder group were used (Ye et al. 2017). The specific number of parental contributors within each mass-cross depended on the physical conditions for spawning and sex distribution within the respective mass-cross (Wang et al. 2008c). In total, 24 mass-crosses were conducted, and the parentage of all individuals produced via these mass-crosses was determined through the application of molecular parentage analysis, utilizing a panel of 10 microsatellite markers (Wang et al. 2008c; Ye et al. 2017). At 90 dph, a phenotypic selection for growth rate was conducted as described in the F_1 population. From each mass-cross, 96–144 F_2 were selected from 3000 selection candidates at 90 dph based on their BLUP breeding values for growth performance, familial origin, and genotypes at the 10 marker loci. From 90–270 dph, the 2800 selected F_2 fish were genotyped with specific markers previously identified in the QTL analysis for growth traits (Wang et al. 2006, 2008a, 2007, 2011; Xu et al. 2006). The body weight of the selected F_2 individuals was recorded at 270 dph. Based on their molecular breeding values (MBVs) and genotype information at loci associated with growth, 600 elite F_2 were selected and later used as F_2 broodfish to produce offspring for further selection. The selected 600 elite F_2 were divided into two lines (e.g., lines 1 and 2, see Fig. 2), each with 300 broodfish based on the genotypic profiles across the 10 marker loci. Notably, significant genetic differentiation was observed between these lines, while concurrently, individuals within each line exhibited genetic dissimilarity from one another. This suggests that the breeding program successfully maintained genetic diversity within the Asian seabass population, even as individuals within the same line were genetically distinct from each other despite being selectively bred for the same targeted traits (Wong et al. 2023a).

From 2014 to 2018, line 1, consisting of 300 F_2 broodfish was used in the selection for growth. Line 2, also comprising 300 F_2 broodfish, was used to select for high omega-3 fatty acid content. To target growth-related traits, six mass-crosses were conducted, involving 15–20 males and 15–20 females selected from the 300 F_2 broodfish. In general, the selection process followed the protocol established for growth selection in F_2. At 90 dph, 1200 elite F_3 were selected from a pool of 18,000 selection candidates. At 270 dph, MAS was carried out using DNA markers identified with QTL (Wang et al. 2006, 2008a, 2011) and association mapping (Xia et al. 2013b; Xu et al. 2006). Ultimately, 200 elite F_3 individuals were selected based on the ranking of their MBV, familial origin, and genotype profiles at the 10 microsatellite loci.

From 2019 to 2023, the 200 elite F_3 were used for mass-crosses to produce F_4 for further selection of growth traits. A total of six mass-crosses were conducted between 15–20 males and 15–20 females selected from the 200 F_3 broodfish. At 90 dph, 1200 elite F_4 were selected from 18,000 selection candidates based on their BLUP breeding values, genotype profiles at the 10 microsatellite loci, and familial origin. At 270 dph, MAS was carried out based on MBVs that were estimated with genotypes at DNA markers associated with growth. A total of 200 elite F_4 were selected to produce F_5 for further selection.

Growth performance of F_1 to F_4 and feed conversion rate

It was reported that the average body weight of F_0 at 90 dph was 26.5 g (Ye et al. 2017). Notably, the body weight at 90- and 270 dph was substantially increased in F_1, F_2, F_3, and F_4 generations (Wong et al. 2023b; Ye et al. 2017; Yue et al. 2017). The detailed data on body weight at 90 dph in the four generations of Asian seabass are shown in Fig. 4. It was reported in our previous paper (Wang et al. 2008c) that the body weight at 270 dph was positively correlated with the body weight at 90 dph. Our recent data showed that the body weight at two years post hatch was also positively correlated with the body weight at 90 dph (Wong et al. 2023a). The feed conversion ratio (FCR) of the improved Asian seabass was between 0.984 and 1.090 (Ngoh et al. 2015), which is similar to that of the improved salmon (*Salmo salar*) (Elvy et al. 2022). This is better than that reported in many other marine aquaculture species, including farmed Chinook salmon (*Oncorhynchus tshawytscha*) (Araujo et al. 2021). Carcass yield is the measure of the proportion of the animal's final live weight yielded after it has been stunned (desensitized), exsanguinated, skinned,

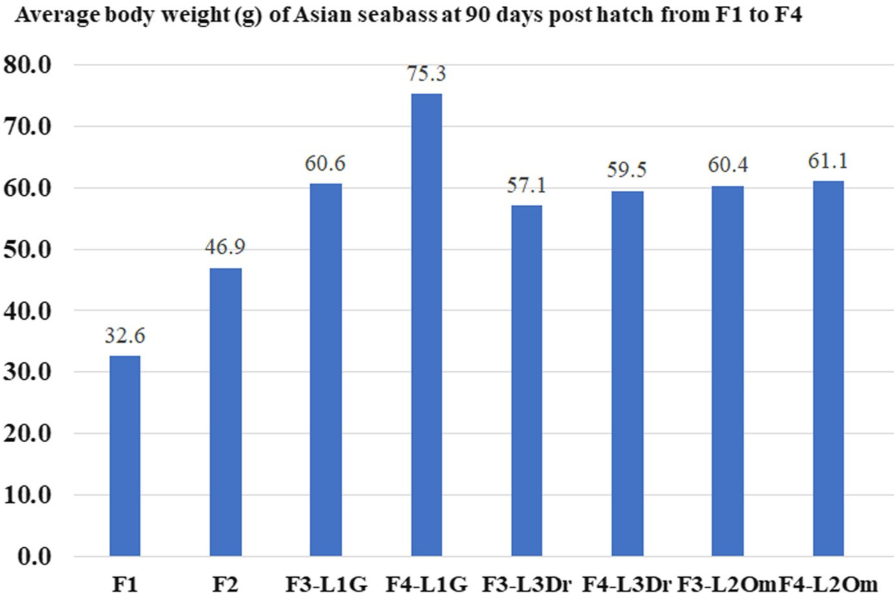

Fig. 4 Body weight (gram) at 90 days post hatch in F_1, F_2, F_3 and F_4 of the Asian seabass. F3-L1G and F4-L1G: Line 1 for growth in F3 and F4; F3-L2om and F4-L2om: Line 2 for high omega-3 contents in F3 and F4; and F3-L3Dr and F4-L3Dr: Line 3 for resistance to three diseases in F_3 and F_4

or scalded, and eviscerated. The average dressing percentage of pigs in the US is approximately 74% (Boler 2014). The carcass yield of the improved Asian seabass at the age of 270 dph was over 80% (our own unpublished data, see Supplementary Table 1), which was higher than that of pig (74%) (Boler 2014) and common carp (66%) (Safner et al. 2001). Therefore, it is certain that in line 1, the growth trait has been substantially improved through breeding over four generations for growth. While a diligent effort was made to maintain consistent aquaculture conditions across the different generations, the observed growth cannot be definitively attributed to genetic factors alone. Ideally, the optimal approach would involve measuring the growth of broodfish from each generation reared under equivalent conditions, thus yielding more precise and dependable growth performance data across different generations. This allows for a direct comparison of growth performance between generations while minimizing the impact of environmental or other factors on the results. However, this practice is not feasible, due to the prohibitively high cost of culture and the long generation interval (4–5 years/generation) of Asian seabass.

Selection for disease resistance

Diseases are a major concern for the sustainability of any aquaculture industry (Yang et al. 2022). Disease outbreaks pose serious challenges for the aquaculture industry. Therefore, selective breeding, coupled with strict biosecurity and effective management practices, is a valuable tool to prevent the introduction and spread of diseases (Ødegård et al. 2011). In the Asian seabass, hatcheries encounter a variety of major diseases, including big belly disease, viral nervous necrosis (VNN) diseases caused by the betairidovirus, the disease caused by iridovirus, parasites, and other bacterial and viral diseases (Hutson 2013). VNN is acute in larvae that are ten days old and often results in 100% mortality (Liu et al. 2016b; Yang et al. 2022). Big belly disease is typically severe in 25- to 60 day-old fry in the nursery, leading to mortality rates spanning from 25 to 99% (Liu et al. 2023). Another disease, caused by an iridovirus, is one of the most severe diseases afflicting tropical marine species and causes acute peaks of mortality of up to 80–90% for larvae in hatcheries (Sun et al. 2020; Wang et al. 2017b). The heritability values associated with disease resistance in aquaculture species commonly range from low to moderate, with a few exceptions (i.e., very high heritability), such as infectious pancreatic necrosis (IPN) disease in salmon (*Salmo salar* L) (Ødegård et al. 2011). Molecular breeding has a huge potential for improving disease resistance through the applications of MAS and GS, while phenotypic selection encounters considerable challenges due to difficulties associated with accurately quantifying disease resistance within farm settings (Yang et al. 2020). In our breeding program, we focused on three diseases, including big belly, and VNN, and the disease caused by an iridovirus.

Selection for resistance against big belly disease in F_2 with molecular parentage

In 2012, outbreaks of the big belly disease (BBD) occurred in two batches of offspring generated by a mass-cross among F_1 broodfish (10–15 males and 10–15 females) in a hatchery (Liu et al. 2023). After the BBD outbreaks, only 437 out of 60,000 F_2 juveniles survived. Ten microsatellite markers were used for a molecular parentage analysis. The parentage analysis showed that four parents (F_1) produced a sizable proportion of young fish which survived the BBD epidemics. Based on growth performance of the surviving offspring at 90 dph, family origin, and allelic variety, 82 BBD surviving offspring (F_2) were chosen. These 82 F_2 fish were from 37 families, which were produced by 8 males and 11 females (Liu et al. 2023), and were used to establish a third line to produce F_3 for selection for disease resistance, including diseases caused by betanodavirus and an iridovirus.

Selection for resistance against NNV and iridovirus in F_3 and F_4

The selection of NNV and iridovirus resistance started in F_3 after the completion of QTL mapping and GWAS for NNV and iridovirus resistance in F_2 and F_3 populations. In line 3, all 82 F_2 broodfish resistant to the big belly disease and with good growth performance were used to generate offspring (F_3) for further selection for NNV and iridovirus resistance. A total of six successful mass-crosses were conducted, each using 15–20 male and 15–20 female broodfish from the 82 F_2 broodfish. For each mass-cross, 60,000 fertilized eggs were collected and cultured under the same conditions as described for the selection for growth. At 90 dph, 192 fastest-growing fish from each mass-cross in F_3 were selected from 3,000 F_3 selection candidates and tagged with PIT tags. These selected F_3 were genotyped with 10 DNA markers to reconstruct pedigrees. From 90 to 270 dph, a total of 1152 preselected fish were genotyped using GBS or PCR-based genotyping as described previously (Wang et al. 2017a). Molecular breeding values (MBVs) of all the preselected 1152 fish were estimated with genotypes at marker loci associated with resistance traits. A total of 200 F_3 with the highest MBVs were selected from different families and used as broodfish to produce F_4 for further selection. For F_4 selection, the same strategy that was used in F_3 selection was applied for selecting F_4. A total of 200 elite brooders with the highest MBVs were selected from 1152 preselected candidates from 6 mass-crosses, each comprising between 10–15 males and 10–15 females.

Performance of disease resistance after selection

Following the selection for resistance against BBD, NNV, and iridovirus in line 3, an assessment was conducted to evaluate the performance of disease resistance. The selected 82 BBD-survival F_2 fish were used as broodfish to produce F_3. These F_2 broodfish produced six batches of offspring over the course of five years without any BBD outbreaks occurring. During the same time, F_2 broodfish that were chosen for growth produced 18 batches of offspring, among which, BBD outbreaks took place in 4 batches of F_3 young fish, with mortality rates ranging from 85.1 to 95.4%. These findings suggest that selecting for BBD survivals from various families during BBD outbreaks with the assistance of molecular parentage analysis to produce offspring, can effectively control BBD epidemics in the next generation. As the causal agent for BBD is unknown and there were no outbreaks of BBD in the F_3 batches, the selection in this line for F_4 was conducted for growth, NNV resistance, and iridovirus resistance.

In our hatchery, no outbreak of disease caused by NNV or iridovirus in the offspring (F_3 and F_4) has been observed since 2014. After challenging offspring (F_3) with NNV in three laboratories (our own lab at Temasek Life Sciences Lab, Professor Yang Daiwen's lab at the National University of Singapore, and Dr Masato Miyata's lab at Temasek Polytechnic, personal communications), no significant mortality ($< 10\%$) was detected as compared with the controls (no virus challenge), although NNV was detected in challenged fish after PCR confirmation. These experimental outcomes for NNV resistance are promising, however, the resistance of the selected fish in the line 3 towards iridovirus has not been tested due to the COVID-19 pandemic from 2020 to 2023. Further experiments involving laboratory-based virus challenges have been planned to determine whether the resistance of line 3 to iridovirus has increased. However, due to the occurrence of COVID-19 from 2020 to 2023, the construction of our pathogen exposure facilities has not been completed. Field tests in other hatcheries could be a more desirable way to examine the resistance to diseases of our improved line.

The average body weight at 90 dph (57.1 ± 15.4 g, $n = 3068$ in F_3, and 59.5 ± 16.9, $n = 1110$ in F_4) in F_3 and F_4 was much higher than that of F_2 (46.90 ± 0.19 g, $n = 12{,}117$). These results indicate that the growth performance of the disease resistant line 3 was improved. This growth increase could be due to the selection for growth at 90 dph followed by the selection for disease resistance using MAS with DNA markers associated with NNV and iridovirus.

Other than the targeted improvement of resistance against the three diseases, there are many other diseases that can reduce the productivity of Asian seabass. For example, from 1992 onwards, outbreaks of scale drop disease (SDD) have been reported in Asian seabass at one year post hatch in Southeast Asia (de Groof et al. 2015). The most striking symptom of SDD is the loss of scales, which is caused by the scale drop disease virus (de Groof et al. 2015). This disease caused a high mortality rate (40–50%) in fish weighing 1–3 kg (Senapin et al. 2019). It was observed in Singapore, Malaysia, and other countries (Nurliyana et al. 2020). It is definite that more diseases will emerge in the Asian seabass aquaculture industry. Therefore, it is essential to further improve the resilience of the Asian seabass towards other emerging diseases. GS and genome editing (GE) might be effective approaches to improving the resilience of Asian seabass, while vaccination for diseases remains an essential component.

Selection for omega-3 contents in F_3 and F_4

Omega-3 fatty acids (omega 3 s) are a key family of polyunsaturated fats (Lin et al. 2019; Wang et al. 2019). There are three main omega-3 s: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found mainly in fish, thus, they are sometimes called marine omega-3 s (Xia et al. 2014). Alpha-linolenic acid (ALA), the most common omega-3 fatty acid in most Western diets, is found in vegetable oils, nuts (especially walnuts), flax seeds, flaxseed oil, leafy vegetables, and some animal fats, especially in grass-fed animals (Stark et al. 2008). The human body generally uses ALA for energy and conversion into EPA, and DHA is comparatively very limited in availability (Burdge et al. 2005). Fish and seafood are rich sources of omega-3 (n−3) long-chain polyunsaturated fatty acids (LC-PUFA), which are highly valued by consumers in developed countries. Eicosapentaenoic (EPA; 20:5n−3) and docosahexaenoic (DHA; 22:6n−3) acids (Tur et al. 2012) have well established benefits in treating cardiovascular and inflammatory diseases and promoting neural development (Calder 2013; Campoy et al. 2012; Delgado-Lista et al. 2012; Gil and Crespo 2012).

MAS for omega-3 contents from F_3

In F_2, QTL and association mapping were conducted to identify DNA markers associated with fatty acids (Sun et al. 2018; Wang et al. 2019; Xia et al. 2014). These DNA markers that were significantly associated with total omega-3, EPH, and DHA were used in selecting elite fish with higher omega-3 content. Briefly, a total of six mass-crosses were conducted to produce F_3 and F_4, each using 15–20 male and 15–20 female broodfish from the 200 F_2 elite broodfish in line 2. For each mass-cross, 60,000 fertilized eggs were collected and cultured under the same conditions as described for selection for growth. At 90 dph, a total of 1,152 fastest-growing fish in F_3 were selected and tagged with PIT tags. These selected F_3 were genotyped with 10 DNA markers to reconstruct pedigrees. From 90–270 dph, these 1152 fastest growing fish were genotyped with markers associated with omega-3 contents (Sun et al. 2018; Wang et al. 2019; Xia et al. 2014). MBVs for each selection candidate were estimated with genotypes at these marker loci and ranked. The top 200 fish with the highest MBVs were selected as broodfish to produce the next generation. Among the 200 selected elite fish, only up to six individuals from a family were chosen to avoid inbreeding.

When compared to the data in F_1 seabass, the omega-3 content in the improved line increased from 650 to 720–760 mg/100 g meat (Wang et al. 2019; Xia et al. 2014), suggesting that MAS is effective in enhancing omega-3 content. The fatty acid composition of fish is influenced by various factors, including diet, salinity, temperature, season, and geographical location, in addition to genetic factors (Tocher 2015). Therefore, to further improve omega-3 content in Asian seabass, both genetic and environmental factors, such as feeds, should be considered.

Genetic diversity in different generations

The long-term success of breeding programs depends on the extent of additive genetic variance in the breeding population (Ambali et al. 1997; Gjedrem 2000). A long-term breeding effort must maintain genetic variety and avoid inbreeding (Gjedrem 2005; Yue et al. 2014; Yue et al. 2009). It is known that reduced genetic diversity in a breeding broodstock results in slow growth in selective breeding programs, such as in the orange-spotted grouper (*Epinephelus coioides*) (Ng et al. 2023). In our Asian seabass breeding program, ten microsatellite markers were used to quantify the genetic diversity in breeding nuclei from the original broodstock (F_0) to the selected fourth generation (F_4). While inbreeding indices were close to zero in F_0, F_1, F_2, F_3, and F_4, the expected and observed heterozygosity were essentially preserved. These findings suggest that inbreeding can be prevented in mass-crosses of genetically unrelated broodfish through selection within families. The effective population size (Ne) declined from 79.6 in F_0 to 30.1 in F_4, and the allele number also declined in the breeding stocks. These findings suggest that the genetic diversity in the breeding program is declining. The non-contribution, unequal contribution of broodstock to offspring, and/or bottlenecks were the causes of the decrease in genetic variety (Wang et al. 2008c; Wong et al. 2023b). Therefore, we advocate adding new Asian seabass individuals from various sources to the breeding nuclei. Our breeding program of the Asian seabass demonstrated that the use of molecular parentage with polymorphic DNA markers and selection within families in mass-crosses was a workable method for lowering the pace of genetic diversity loss and preventing inbreeding in breeding operations.

Commercialization of improved Asian seabass

In March 2018, a company named Allegro Aqua Pte Ltd (AA) was established with the objective of commercializing hybrids resulting from the crossbreeding of different elite Asian seabass lines, thereby promoting the growth of aquaculture production. AA was established by Temasek Life Sciences Laboratory (TLL), with support from Temasek Life Sciences Accelerator (TLA). AA has worked with various farms and demonstrated that they could scale up the production of the genetically improved Asian seabass with better growth and survival rates. Due to the promising performances in local and regional farms, AA was acquired by the world's largest Asian seabass producer, Barramundi Asia Pte Ltd (BA) (https://barramundi.com/). BA has farmed Asian seabass in the ocean and draws on world-class sustainable fish farming practices and aquaculture technology in Singapore, Australia, and Brunei (Merican 2020b). It developed an ocean farm in Brunei's Nankivell Offshore Aquaculture Site. BA also operates its own Recirculating Aquaculture System hatchery, nursery, and deep-sea cage "grow-out" farms (Merican 2020a). The Barramundi Group was listed on the Euronext Growth Oslo exchangelo, Oslo, Norway, in August 2021.

Challenges in the Asian seabass breeding program and solutions

Although the 20 year breeding program successfully improved growth rate, omega-3 content, and resistance to diseases such as big belly disease, VNN, and iridovirus, the breeding program encountered several challenges.

Sex reversal is very common in Asian seabass (Jerry 2013). This fish typically reaches sexual maturity as a functional male between the ages of three and four years before transitioning to a female during the following spawning seasons (Roberts et al. 2021). As such, sex reversal leads to the production of more female populations. If all the selected broodfish in one generation are of similar age, there may not be enough males available to fertilize the females during subsequent spawning. To mitigate this, the age of broodfish in each generation should span two to three years. Recently, it was found that the genetically improved Asian seabass change sex at an earlier stage in development compared to the wild-type Asian seabass (Roberts et al. 2021). Therefore, it is essential to find ways to prevent the males from transitioning into females. Although numerous methods are available to inhibit sex reversal (Pandian et al. 2003; Tenugu et al. 2022), the optimal approach for Asian seabass has yet to be determined. On the other hand, studying the mechanisms underlying sex degermation, differentiation, and reversal (Li et al. 2004; Wang et al. 2022) may yield new insights on sex reversal.

Our findings revealed an uneven contribution of broodfish to their offspring. While mass-crosses facilitated the generation of genetically diverse offspring by producing numerous parental combinations (Loughnan et al. 2013), certain broodfish were observed to not produce any offspring in certain cases (Wang et al. 2008c). The uneven and non-contribution of broodfish to offspring resulted in a reduced effective population size (Wong et al. 2023b). Therefore, assessing the reproductive readiness of broodfish is crucial for ensuring their effective contribution to offspring production. In general, broodfish selected for spawning must be confirmed to contain sperm to ensure their suitability. Hormonal induction of spawning and artificial insemination could be an effective way to ensure the contribution of broodfish to offspring production (Berlinsky et al. 2020), however, it could be labour-intensive.

Without knowledge of the exact causal agent responsible for the big belly disease (BBD), it remains challenging to definitively conclude the genuine resistance of the improved line to this disease, despite the successful selective breeding for BBD (Liu et al. 2023). Thus, it is essential to identify the pathogen/pathogens that cause BBD. The identified pathogen or pathogens could be used to challenge selected fish to examine their survival rate. In addition, it would be possible to conduct a GWAS using pathogen-challenge experiments to enable GS for BBD resistance.

MAS and GS have been applied to improve growth, resistance to NNV and iridovirus, and omega-3 content. It is worth noting that the accuracy of both MAS and GS depends highly on the degree of relationship between the training population and selection populations, and this accuracy is not consistent across generations (Boudry et al. 2021). The MAS and GS models must be further improved in the coming generations of the selective breeding program by integrating more DNA markers associated with these traits, which should be identified in QTL mapping and GWAS for traits of interest. To improve genomic prediction in genetically distant populations, identification and utilisation of causative variants and/or markers in linkage disequilibrium with traits is essential (Yang et al. 2022). For a typically polygenic trait, this is likely to require very large reference population sizes that are genotyped at high density or fully sequenced (Hickey et al. 2013). Strategic investment in the collection of large-scale genetic datasets facilitates the identification of candidate genes and mutations that are suitable for functional testing, enabling a deeper understanding of the underlying biology of economically important traits. Further improvement of the assembly and functional annotation of the Asian seabass reference genome (Vij et al. 2016) will be key to prioritising putative causative variants.

It is very common for a reduction in genetic variation to occur in a breeding program due to selection and bottlenecks. Therefore, preserving genetic variation and mitigating the rate of reduction of genetic diversity are crucial to ensuring the success of a breeding program. In our Asian seabass breeding program, inbreeding was controlled and allelic diversity was reduced by using mass-crosses with genetically distant broodfish, molecular parentage, and within family selection (Wong et al. 2023b). To improve allelic diversity, in addition to the technologies applied in this breeding program, the introduction of individuals from the wild in Southeast Asia or Australia is necessary. Genotyping of the introduced individuals using developed DNA markers (Yue et al. 2009; Zhu et al. 2006, 2010) can be performed to determine their relationship with broodfish in the breeding program, as well as to assess the presence of beneficial genotypes for important traits.

Although the three improved lines have been verified with our own experiments and small-scale commercial production, they have not been extensively tested by other Asian seabass farms in Southeast Asia or other continents. Therefore, more field tests in commercial farms should be conducted to test the performances of the three improved lines under different culture conditions (e.g., lower and higher dissolved oxygen levels in water) and systems (e.g., nets in the sea, ponds, and land-based recirculating aquaculture systems).

Since the three lines of Asian seabass from the breeding program have been genetically improved for growth, omega-3 content, and resistance against big belly disease, nodavirus, and iridovirus, it is possible that their altered genetic profiles may affect their nutritional requirements. Therefore, it is essential to determine their specific nutritional requirements and develop tailored feed compositions for these improved lines.

It is known that climate change has affected aquaculture to various extents depending on climatic zones, geographical areas, and culturing systems (Galappaththi et al. 2020). The improved Asian seabass, which has adapted to the environmental conditions in Singapore, may not be suited for culture conditions in other regions. Climate change may lead to outbreaks of existing and new pathogens or parasites. In addition, it is also well known that a limited supply of fishmeal would affect future increases in global aquaculture production (Olsen et al. 2012). To address these challenges, it is critical to breed a new, robust line of Asian seabass (Fig. 5) that is highly resistant to both current and emerging diseases and stressors, while also being adaptable to a wider temperature range. With careful planning, selective breeding, and regular monitoring, it is possible to develop a fish population that is well-suited to environmentally sustainable feeds that use reduced fishmeal. To facilitate commercial production, it is recommended that farms utilize the hybrids generated by crossing individuals from different lines.

Breeding is a lengthy and costly process to generate long-lasting beneficial effects on aquaculture production (Gjedrem 2005; Gjedrem et al. 2012). Therefore, policymakers and funding agencies should provide incentives to boost selective breeding programs. It is essential to protect the intellectual property (IP) of the improved Asian seabass. It has been shown that hybridization provides plant breeders and aquaculture breeding companies with significant advantages over grow-out farmers (Kingsbury 2011; Rahman et al. 2018). Therefore, hybridization between improved Asian seabass lines may be an effective mechanism for protecting IPs generated in

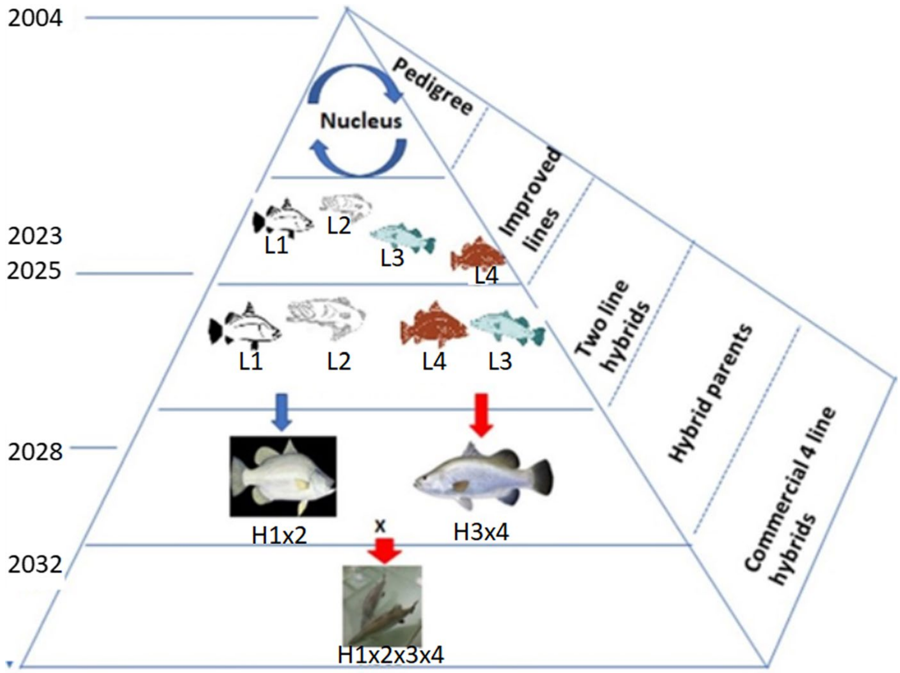

Fig. 5 Status and future directions of the Asian seabass breeding program started in 2004 in Singapore. From 2023 on a fourth line for robustness will be established using conventional and molecular breeding approaches. For commercial production, hybrids generated between lines will be used while the four lines will be further improved with molecular breeding approaches

the 20 year breeding program. The importance of new breeding technologies in Asian seabass production is increasing and is expected to play a significant role in the further advancement of the Asian seabass aquaculture industry.

Conclusions and future perspectives

The 20 year Asian seabass program used a founder population of 549 broodfish with high genetic variation and generated many genomic resources to accelerate the improvement of growth, resistance to diseases, and omega-3 fatty acid content. The establishment of three elite lines, consisting of approximately 200 broodfish per line per generation, for commercial production has been made possible through the application of molecular parentage, within-family selection, MAS, and GS. While the breeding program has shown success in improving growth, omega-3 content, and resistance against three major diseases, we have observed a reduction in effective population size and allelic diversity, despite the absence of inbreeding risk. The reduction of the effective population size was mainly caused by non-contribution and uneven contribution of broodfish to offspring production. To ensure continuous improvement of important traits and adaptability to climate change, it is essential to introduce broodfish from diverse sources and to breed a new line of Asian seabass for robustness using QTL mapping, GWAS, MAS, GS, and GE. With further improvement of the three existing elite lines and the development of a new line for robustness, it is anticipated that soon, four-line hybrids will be created and employed for commercial production. Simultaneously, the four improved lines will be subjected to further refinement utilizing novel and emerging technologies (Yue et al. 2022) (Fig. 5). The improved broodfish in each line will be transferred to multiplier stations to provide sufficient commercial seed for production. Considering the current data on the seed market and the trend of producers merging into larger companies, it may become necessary for the largest marine fish hatcheries to adopt a multiplier strategy. We are confident that the genetically improved Asian seabass will become an economically significant species for aquaculture on a global scale. The knowledge acquired from this breeding program and the lessons learned from it are valuable for those contemplating the initiation of breeding programs for other aquaculture species. For resource-limited countries, reducing the cost of selective breeding in aquaculture requires innovative strategies and approaches, such as collaborative research and knowledge sharing. The key to reduce the cost of breeding programs is the fostering of collaborative networks, sharing of resources, and prioritization of traits that offer the greatest benefits.

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Data availability All data supporting the findings of this study are available in cited papers in article and its supplementary information files.

Code availability Not applicable**.**

Declarations

Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Ethical approval Not applicable**.**

Consent to participate Not applicable**.**

Consent to publication We give our consent for the publication of identifiable details, which can include figure(s) and/Statements and Declarations.

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